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 Escola Superior de Biotecnologia


**Biotechnological tools in *P. pinaster*
 to fight against *Bursaphelenchus
 xylophilus***

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 January 13th 2011











Forests


- Forests and sustainable development
- 1.6 billion depend on forests for their livelihoods
- Habitats to two-thirds of all species on earth
- Deforestation, fires and...

... pinewood nematode




Outline

1. Identification of resistant genotypes
2. Identification of resistance locus (QTLs)
3. Plant immunization: study of a non virulent isolate
4. Identification of resistance genes
5. Genetic transformation of *P. pinaster*

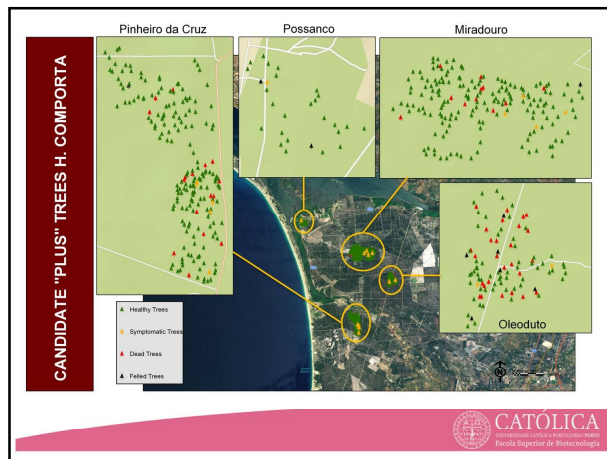


Identification of resistant genotypes



Susceptibility/resistance evaluation

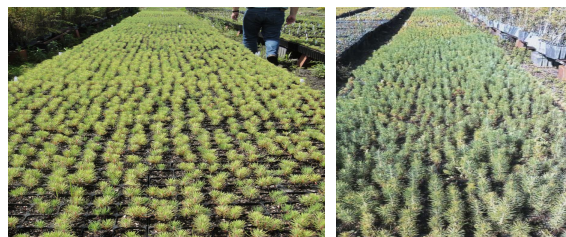
Locations at Herdade da Comporta	N ^{er} of live selected trees		
	August 2010	July 2010	November 2010
Oleoduto	97	64	64
Possanco	28	26	25
Miradouro	182	168	165
Pinheiro da Cruz	197	178	176
Total	504	436	430



Susceptibility/resistance evaluation



Nursery

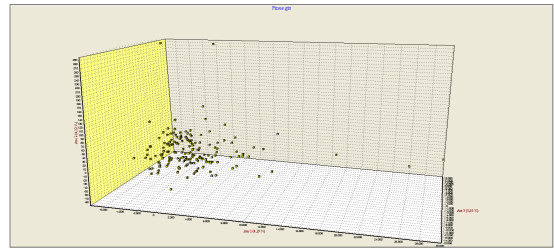


April 27th, 2010

September 21st, 2010

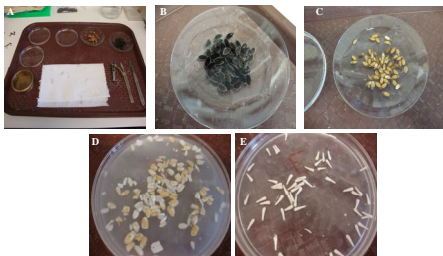
Identification of resistance locus (QTLs)

Genotypical characterization of selected trees

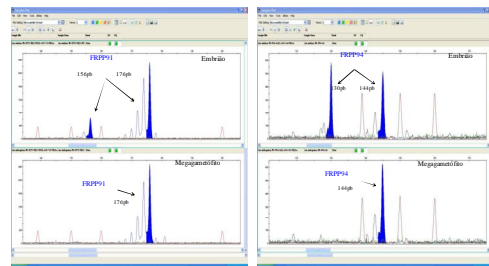


Microsatellites: 175
AFLP: 121

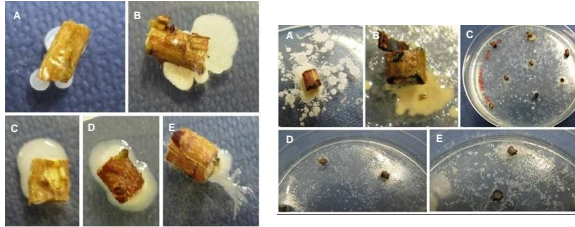
Genotypical characterization of clones belonging to the tested clonal nursery and offspring



Genetic profiles from embryo and megagametophyte



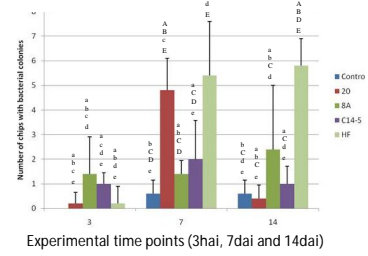
Bacterial colonies



Bacterial colonies in the chips inoculated with virulent nematode isolate 8A (A and B), with virulent nematode isolate 20 (C and D), and bacterial colonies along the trails of avirulent nematode isolate C14-5 (E), 3hai.

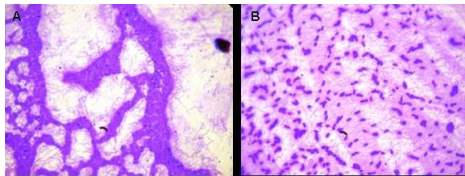
Figure (A-E): Bacterial colonies along the trails of virulent nematode isolate 8A (A-C) and HF (D and E), 14dai.

Number of inoculated chips with bacterial colonies

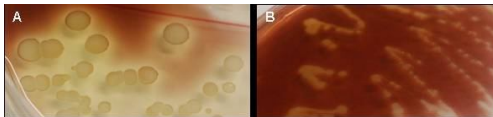


Experimental time points (3hai, 7dai and 14dai)

Bacterial classification-classical methods

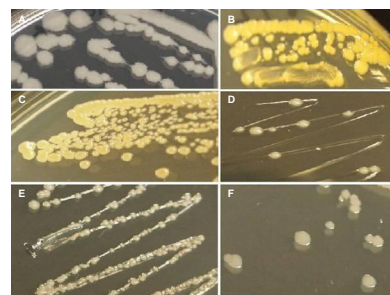


Flagella stain for bacterial colonies 5 (A) and 24 (B).



Positive (A) and a negative (B) result for starch hydrolysis test

Bacterial classification-classical methods



Example of bacterial colonies isolated from inoculated twigs and grown on NA medium showing different morphologies.

Bacterial classification-molecular methods

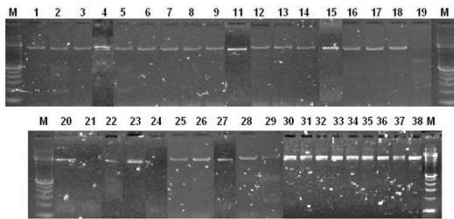
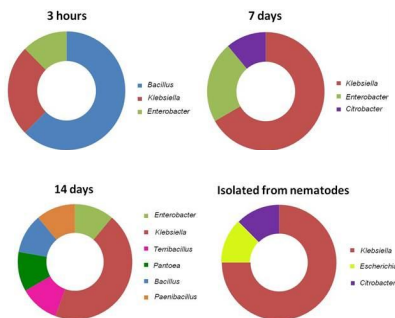


Figure: Agarose gel stained with SYBR® Safe DNA gel stain showing the amplification of the 1500 bp fragment corresponding to the 16S rRNA gene (M indicates the marker ladder; columns 1 to 38 represent the isolated colonies).

Bacterial classification-molecular methods

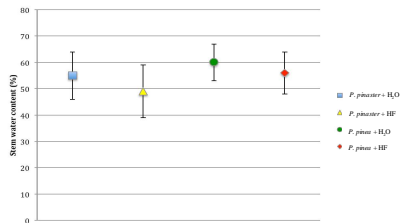
Sample	Treatment	Experimental time point	Associated bacterial species	Sample	Treatment	Experimental time point	Associated bacterial species
22	8A	3h	<i>Bacillus megaterium</i>	27	20	3h	<i>Bacillus simplex</i>
23			<i>Bacillus pumilus</i>	1			<i>Klebsiella oxytoca</i>
25			<i>Bacillus megaterium</i>	2			<i>Klebsiella oxytoca</i>
26		<i>Klebsiella oxytoca</i>	3	<i>Enterobacter cloacae</i>			
4		7d	<i>Klebsiella oxytoca</i>	16		14d	<i>Klebsiella oxytoca</i>
18		14d	<i>Klebsiella oxytoca</i>	17			<i>Paenibacillus tundiae</i>
20	IN*	<i>Paenibacillus tundiae</i>	35	IN*	<i>Klebsiella oxytoca</i>		
37		<i>Klebsiella oxytoca</i>	36		<i>Escherichia coli</i>		
38		<i>Klebsiella oxytoca</i>	28		<i>Bacillus megaterium</i>		
30	HF	3h	<i>Enterobacter cloacae</i>	29	C14-5	3h	<i>Klebsiella oxytoca</i>
7			<i>Enterobacter cloacae</i>	5			<i>Citrobacter freundii</i>
8			<i>Klebsiella oxytoca</i>	6			<i>Klebsiella oxytoca</i>
9		7d	<i>Klebsiella oxytoca</i>	14		14d	<i>Klebsiella oxytoca</i>
11		14d	<i>Terribacillus stanssens</i>	31		IN*	<i>Klebsiella oxytoca</i>
12		<i>Klebsiella oxytoca</i>	32	<i>Klebsiella oxytoca</i>			
13	<i>Enterobacter oryzae</i>	15	Ø	14d	<i>Bacillus megaterium</i>		
33	IN*		<i>Citrobacter freundii</i>				
34			<i>Klebsiella oxytoca</i>				

Summary of identified bacterial genus

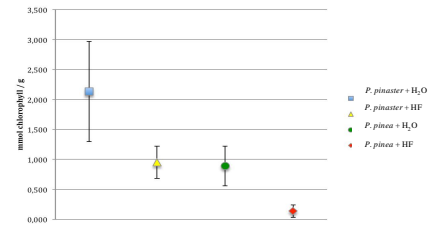


P. pinaster vs. *P. pinea*

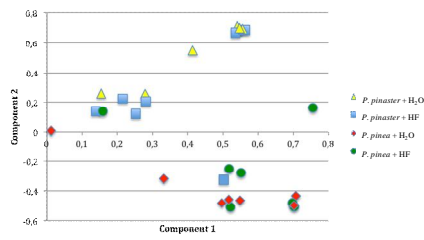
Stem water content of *P. pinaster* and *P. pinea*



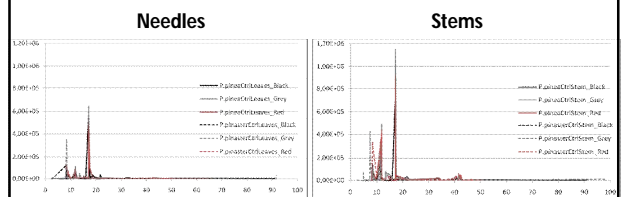
Chlorophyll of *P. pinaster* and *P. pinea*



Volatiles in *P. pinaster* and *P. pinea*



Volatiles in *P. pinaster* and *P. pinea*



Identification of resistance genes

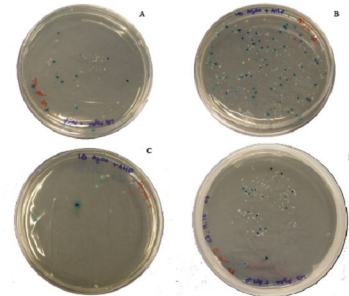


Figure 11 - Blue/white screening for target clones. A total of 50 µl of cloning product were plated on Amp^rGal selective plates. A) Unsubtracted Tester, B) Unsubtracted Driver, C) Forward Subtraction, D) Reverse Subtraction.

Genes expressed by *P. pinaster*

Seq.	Size (bp)	Protein/ORA similarity	Origin of the filter sequence	Hit # I		Hit # II	
				Ass No. of similar sequences	Similarity	Ass No. of similar sequences	Similarity
U1-1	864	Unknown	<i>Pinus edulis</i>	AB234386.1	11-137 (99%, 510 aa)	32-38494.1	0.0 (0%, 122 bp)
U1-2	865	SRF1, domain 14-57M	<i>P. pinaster</i>	BM12681.1 (P38121)	39-141 (97%, 103 aa)	89-29725.1	46-232 (100%, 461 bp)
U1-3	869	ADP-350-like (PFLA2)	<i>Pinus resinosa</i>	XP_023368349.1	11-146 (99%, 449 aa)	83-8161.1	0.0 (0%, 1336 bp)
U1-4	930	-	-	-	-	-	-
U1-3	151	Cytochrome b	<i>Pinus edulis</i>	AAJ59889.1	7-86 (97%, 139 aa)	AT32123.1	86-132 (92%, 477 bp)
U1-6	869	Unknown	<i>Pinus attenuata</i>	AD227504.1	26-33 (95%, 674 aa)	87-134695.1	0.0 (0%, 1132 bp)
U1-9	538	Unknown	<i>Pinus massoniana</i>	AF381393.1	16-33 (96%, 133 aa)	77-104323.1	31-163 (92%, 888 bp)
U1-8	865	Transcription factor 1-like	<i>Pinus edulis</i>	CAC22735.1	11-149 (97%, 444 aa)	87-13462.1	0.0 (0%, 1872 bp)
U1-9	603	Transcription factor 1-like	<i>P. pinaster</i>	AC288133.1	11-108 (97%, 318 aa)	AT441305.1	0.0 (0%, 5708 bp)
U1-10	865	Unknown	<i>Pinus edulis</i>	AB183223.1	31-63 (97%, 130 aa)	3267-8404.1	11-48 (95%, 829 bp)
U1-11	869	Unknown	<i>Pinus glauca</i>	AF381393.1	36-71 (96%, 307 aa)	87-134648.1	0.0 (0%, 1666)

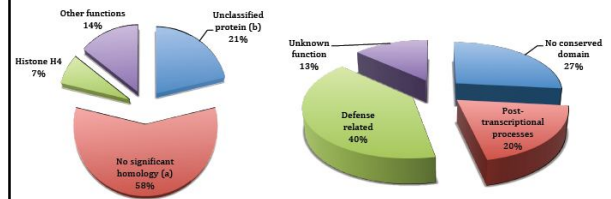
Genes expressed by *P. pinea*

Seq.	Size (bp)	Protein/ORA similarity	Origin of the filter sequence	Hit # I		Hit # II	
				Ass No. of similar sequences	Similarity	Ass No. of similar sequences	Similarity
U1-1	866	Unknown	<i>Pinus edulis</i>	AB234386.1	36-36 (99%, 510 aa)	32-38494.1	0.0 (0%, 117 bp)
U1-2	867	Unknown	<i>Pinus glauca</i>	-	-	87-116783.1	61-62 (99%, 7745 bp)
U1-3	868	Chloride channel	<i>Pinus glauca</i>	AF273731.1	11-138 (97%, 598 aa)	DQ437297.1	0.0 (0%, 2197 bp)
U1-4	863	Unknown	<i>Pinus glauca</i>	AB233531.1	11-126 (97%, 588 aa)	87-116780.1	6.0 (96%, 1293 bp)
U1-5	307	SRF1-like	<i>P. pinaster</i>	-	-	AT32123.1	1.01-102 (99%, 248 bp)
U1-6	703	Unknown	<i>Pinus edulis</i>	AB232231.1	31-76 (97%, 160 aa)	87-13462.1	1.01-102 (99%, 943 bp)
U1-7	866	W14-like protein	<i>Pinus massoniana</i>	3-BA00208.1	140 aa	AB233613.1	31-62 (95%, 614 bp)
U1-8	819	Unknown	<i>Pinus glauca</i>	AB234618.1	11-49 (97%, 819 aa)	87-116781.1	0.0 (0%, 1197 bp)
U1-8	311	Transcription factor 1-like	<i>P. pinaster</i>	AC091103.1	31-53 (97%, 137 aa)	AT32123.1	0.0 (0%, 541 bp)
U1-10	311	CysC	<i>Pinus massoniana</i>	AC113383.1	11-33 (96%, 137 aa)	87-13462.1	1.01-102 (98%, 474 bp)
U1-11	783	Unknown	<i>Pinus glauca</i>	AF273731.1	11-138 (97%, 598 aa)	87-116783.1	0.0 (0%, 1340 bp)
U1-12	413	Unknown	<i>Pinus edulis</i>	AB233139.1	93 aa	32-38494.1	0.0 (0%, 654 bp)
U1-13	442	Unknown	<i>Pinus edulis</i>	AB233531.1	11-33 (97%, 60 aa)	32-38494.1	31-34 (98%, 443 bp)
U1-14	863	Unknown	<i>Pinus edulis</i>	AB233531.1	348 aa	32-38494.1	31-34 (98%, 1315)
U1-15	869	Asparagine lyase	<i>P. pinaster</i>	-	-	87-13462.1	31-34 (98%, 1666)

Genes differentially expressed in *P. pinea*

Protein/DNA similarity	Origin of similar sequence	Acc No. of similar sequence	Eblast X Similarity	Acc No. of similar sequence	Eblast X Similarity
Histonas 194	Histonas 194	AF053136.1	76-98 (94%, 108 aa)	XP_021186.1	84-84 (99%, 612 bp)
Histonas 194	Histonas 194	FS609721.4_P2.L1.C	*	XP_021186.1	84-84 (99%, 612 bp)
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Histonas 194	Histonas 194	NP_031077477.1	86-18 (74%, 85 aa)	BT112197.1	86-80 (74%, 612 bp)
Histonas 194	Histonas 194	XP_021186.1	46-82 (81%, 109 aa)	BT112197.1	86-80 (74%, 612 bp)
CU17461-23a (249)	Histonas 194	XP_032888736.1	26-84 (80%, 138 aa)	BT112197.1	18-38 (72%, 914 bp)
.
.

Expressed genes



Transformation of *P. pinaster*

Embryogenic lines

Initiation media	Family		
	F49	F68	F82
M1	15	53	45
M2	9	27	7
M3	49	71	14
M4	29	48	8
M5	12	26	6
M6	34	57	12
Total N° embryogenic lines/family	148	282	92

Table: Number of embryogenic lines that were established from 3 families in the different initiation media.

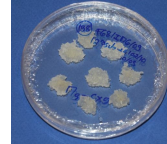
Embryogenic lines

Maturation type	N ^o of embryogenic lines
E	179
+	59
++	37
+++	45

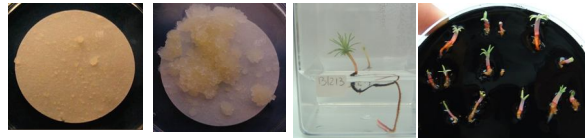


Somatic embryos at different developmental stages obtained from embryogenic lines in maturation conditions.

Embryogenic line in proliferation conditions



Embryogenic cultures in re-establishment and germinating media



Conclusions

- Seeds of 113 surviving trees were collected for confirmation of resistance
- Microsatellites and AFLPs generated high polymorphism
- DNA extraction protocol for megagametophytes is suitable
- C14-5 seems less virulent in *P. pinaster*
- At na early infection stage, *P. pinea* and *P. pinaster* have different physiological, morphological and metabolic responses
- Probable resistance genes identified (majority were defence-related)
- Embryogenic *calli* successfully produced with regeneration potential.

Future prospects

- Establishment of clonal seed orchard at Viveiro Florestal de Valverde
- Continue genotyping the 46 clones from the breeding population
- Genotype selected trees from central Portuguese region (mata do Buçaco)
- Transcriptomics:
 - 454 sequencing of *P. pinea* and *P. pinaster* (mRNA sent already)
 - Confirmation of SSH results with Q-RT PCR
- Tree immunization:
 - Repeat inoculation studies with C14-5
 - Inoculate trees with asseptical nematodes
- Metabolomics:
 - Identification of the head space volatiles differentially released using GC-MS
- Integrate all generated data in a phenomics network

Acknowledgments

- MADRP, IFAP I.P., AFN, FORESTIS
- Collaborators: team INRB, team IBET, Hajime Kosaka, Dr. Aikawa, Dr. Manuel Mota
- **Plantech lab**



Susceptibility screening for other trees

Sampling times for the preliminary assay

